Identification and characterization of the novel tumor suppressor gene *gon-14* in *C.briggsae*.

Research Thesis

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By

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Abstract

This project uses the nematodes *C.elegans* and *C.briggsae* as simple experimental systems to identify new tumor suppressor genes and to understand how these influence cell signaling and cell division. Comparisons between the two species will allow us to better understand how genetic backgrounds can influence the function of tumor suppressor genes. Tumor suppressor genes normally prevent inappropriate cell division and loss-of-function mutations in these genes are associated with cancer. Our lab has identified new mutants in *C.briggsae* that exhibit a multivulva phenotype and in preliminary results I have found that one of these genes is *Cbr-gon-14*. In *C.elegans, gon-14* encodes a nuclear protein that influences gonad development but has only a modest impact on vulva cell development. Thus, we have identified that this is a novel tumor suppressor gene that influences cell division differently in the distinct genetic backgrounds represented by the two species. To identify the mutation associated with the *C.briggsae* mutant allele, I completed DNA sequencing studies using the *C.briggsae* mutant strain we had in the lab. I found two alleles for the *C.briggsae gon-14* gene. I completed cDNA amplification of the entire gene to verify the structure of the gene. In addition, I also completed a cDNA amplification of the *Cbr-gon-14* worms grown at 25°C. These animals showed the mutation that was expected for the *Cbr-gon-14* mutants. I also completed a temperature shift experiment to determine the temperature sensitive period of the *Cbr-gon-14* mutants. The results of these studies aid in the identification and genetic characterization of a new tumor suppressor gene in *C.briggsae* animals. Comparing the genetic backgrounds between the *C.elegan* and *C.briggsae* gon-14 gene will aid in the understanding of how different genetic backgrounds influence the function of tumor suppressor genes.

Introduction

This project focuses on the study of vulva development in nematodes *C.elegans* and *C.briggsae* to understand and compare the molecular networks between the two species. We use these two nematode species because they both exhibit similar patterns of development, morphogenesis and cell differentiation resulting in similar phenotypic outcome (Gupta et al. 2007; Zhao et al. 2010; Sharanya et al. 2012). However, the genetic pathways underlying this similar phenotypic outcome between the two species are not well understood.

In both *C.elegans* and *C.briggsae*, the vulva is made from the division of Vulval Precursor Cells (VPC's). There are three types of cell fates that are produced once the anchor cell (AC) initiates the cell signaling. There is primary cell fate (1°), secondary cell fate (2°) and tertiary cell fate (3°). The cell just below the anchor cell, P6.p, adopts a primary cell fate. The two cells neighboring P6.p, -P5.p and P7.p- become secondary cell fate. The rest of the cells, P3.p, P4.p, and P8.p, become tertiary cell fate (Figure 1).

The way this egg-laying structure works is that the Anchor Cell secretes a signal (LIN-3/EGF) that activates a cascade of events in the VPC's resulting in the vulva development. The LIN-3/EGF (Epidermal Growth Factor) and LIN-12/Notch pathways are important pathways that regulate cell division and cell growth. These pathways are conserved in nematodes, and are important for regulating cell division of ventral epithelial cells during development of the vulva (Figure 2). The LIN-3/EGF pathway mediates induction of the nematode vulva (Sternberg and Horvitz, 1991) and the LIN-12/Notch pathway mediates lateral signaling between vulva precursor cells to allocate precursors to specific vulva fates (Greenwald et al., 1983).

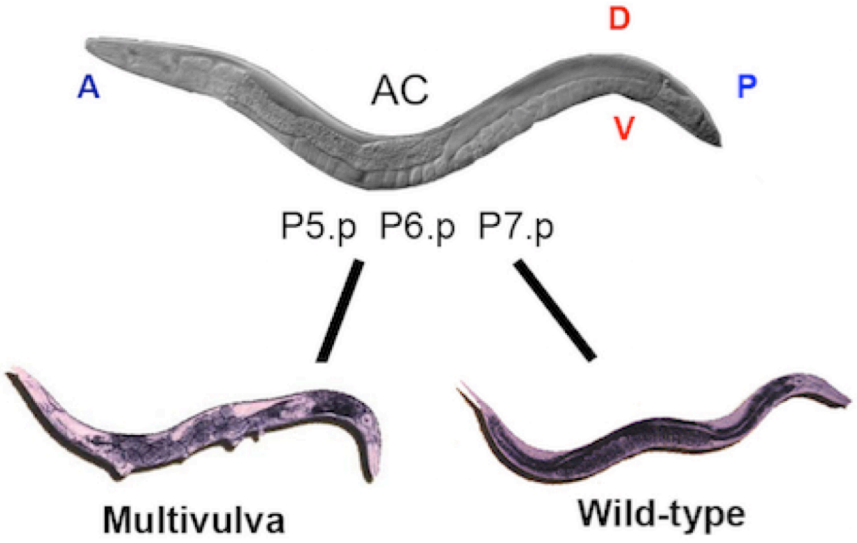

Figure 1: Shows vulva development in both *C.elegans* and *C.briggsae* and the phenotypic differences between wild-type and multivulva animals (Weidhaas, J. 2006)

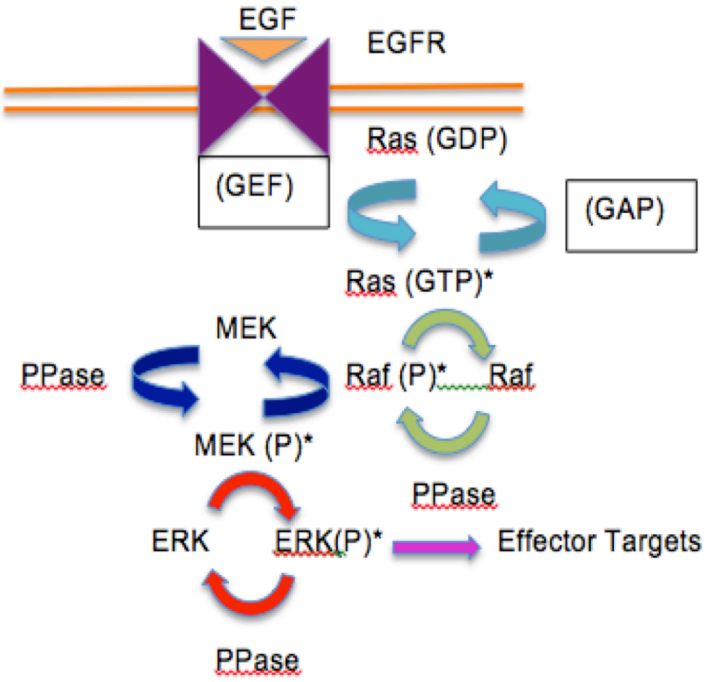

Figure 2: The LIN-3/EGF signaling cascade regulates cell division and growth in both *C.elegans* and *C.briggsae* animals.

Mutations within the LIN-3/EGF cell-signaling pathway can change the phenotype of the animal. My research focuses on mutants that show the Multivulva (Muv) phenotype. Mutant worms that show the Muv phenotype have more VPC's dividing than normal (Figure 1). Animals showing the Muv phenotype exhibit inappropriate division of distal VPC's and therefore these mutants identify tumor suppressor genes (Sharanya et al. 2015).

Our lab has identified new mutants in *C.briggsae* that exhibit this Muv phenotype. My research objective is to identify and characterize one of these mutant genes represented by the alleles gu102 and gu168. I show that these mutations are in the *C.briggsae gon-14* gene. This work argues that gon-14 is a novel tumor suppressor gene in nematode species *C.briggsae*.

The *C.briggsae gon-14* gene is an ortholog of the *C.elegans gon-14*. In *C.elegans* the gon-14 gene is involved in gonad development, muscle contraction and multicellular organism growth, among other functions (Mulder et al., 2013). Loss of function of this gene causes multiple phenotypic expressions such as Muv defects and temperature sensitive larval arrest (Chesney et al., 2005).

When we compared the VPC induction between *C.elegans* and *C.briggsae* gon-14 mutants, average induction is higher in *C.briggsae* animals arguing that the EGF signaling network in *C.briggsae* is more sensitive than in *C.elegans* (Figure 3) (Sharanya et al. 2015).

Genotype	% Induction of VPCs						Average induction	# animals examined
	P3.p	P4.p	P5.p	P6.p	P.7p	P8.p		
Wild type	0	0	100	100	100	0	3.0+/- 0.0	30
C.elegans gon-14 mutants	17	23	96	100	100	40	3.8+/-0.1	54
C.briggsae gon-14 mutants (gu102)	14.8	87.5	99.5	100	100	92.6	4.9	108

Figure 3: Comparison of the percent induction of Vulva Precursor Cells between *C.elegans gon-14* mutants and *C.briggsae gon-14* mutants (Sharanya et al. 2015).

To further understand this novel tumor suppressor gene I did a complete DNA sequencing analysis using the *C.briggsae* mutant strains we had in the lab. Then, to corroborate the entire gene structure I completed a full cDNA amplification of the entire gene. In addition, I completed temperature shift experiments to identify the temperature sensitive period of these mutants since previous studies suggest that *C.briggsae gon-14* mutants are temperature sensitive. Altogether, the results of this research will help the understanding of the novel tumor suppressor gene *gon-14* in *C.briggsae* animals.

Materials and methods

Strains

The Multivulva mutants used in these experiments were generated from a genetic screen by mutagenizing AF16 animals in a 25mM M9 buffer solution of Ethyl Methane Sulfonate (EMS) (Sharanya 2015). Dr. Helen Chamberlin generated the *C. briggsae* gu102 and gu168 mutant strains from a genetic screen. The *C.briggsae* wild-type strain used was AF16.

DNA sequencing

I used DNA sequencing methods to determine the DNA mutation associated with particular mutant candidates *Cbr-gu102* and *Cbr-gu168*. To find the mutation in the two candidates I prepared genomic DNA from each strain. Mixed staged animals were harvested using standard methods (Brenner 1974). Then I used PCR to amplify sections of the *gon-14* gene from the mutant DNA. Samples were digested using pBII (pBluescript) as the cloning vector, and the XhoI and SacI enzymes were used. Samples were then heat inactivated, the vector was dephosphorylated and samples were ligated and transformed. The Plant-Microbe Genomics Facility at The Ohio State University sequenced the PCR products.

DNA sequencing using the Sanger method: Sanger sequencing technique is one of the most commonly used methods for DNA sequencing of recombinant plasmids. Employing the Sanger Sequence protocol involves using DNA polymerase, designed primers (forward and reverse primers), unlabeled deoxynucleotide triphosphates (dNTPs) and the template DNA (NEB, 2016).

- DNA was isolated from the samples of interest.

- Then we amplified the DNA region of interest by using PCR. This region of interest was selected from primers that I designed with Dr. Chamberlin's assistance. These primers are listed on Table S1.
- Products were generated by cycle sequencing and then purified using QIAquick PCR Purification kit and manufacturer's protocol (QIAGEN, 2013).
- Products were analyzed using gel electrophoresis.

cDNA amplification

C.briggsae gon-14 cDNA was sequenced to identify exon boundaries. RNA was extracted from animals in mixed stages grown at both 20°C and 25°C using the TRIzol Reagent (Invitrogen, USA). RNA concentration was measured using absorption spectrometry. mRNA was reverse transcribed using random primers and cDNA was amplified using PCR. For cDNA synthesis, 1ul of RNA was reverse transcribed by using SuperScript III Reverse Transcriptase (SSIII) following the manufacturer's instructions (Invitrogen, USA). RT-PCR products were purified and then sequenced. *C.briggsae gon-14* animals grown at 25°C were sequenced to determine if the temperature affected the mutation. The primers used for the cDNA amplification are listed on Table S1.

TRIzol Reagent Method (Invitrogen, USA): RNA was extracted following the manufacturer's instructions.

RNA collection: 3 units of TRIzol were added for every 1 unit of sample. Samples were frozen at -80°C for at least 3 hours.

Phase separation: chloroform was added, samples were incubated and centrifuged. The upper aqueous phase was pipetted out. The aqueous phase was used for the next stages.

RNA precipitation: Isopropanol was added, samples were incubated at room temperature and centrifuged at 4°C.

RNA wash: The supernatant was removed and the RNA pellet was left. The pellet was washed with 75% ethanol. Samples were vortex, centrifuged at 4°C and the wash was discarded. RNA pellet was air dried.

RNA resuspension: The RNA pellet was resuspended in RNase-free water and incubated in a water bath. RNA was stored at -80°C.

Temperature Shift Experiment

Previous data from Dr. Chamberlin's laboratory shows that the *C.briggsae gu102* mutant strain is temperature sensitive (Figure 4). Gravid adult worms were treated with bleach to isolate embryos (Lewis and Fleming, 1995). The embryos were incubated in M9 buffer without food (Brenner, 1974) at room temperature for 16-18 hours for AF16, and 22-25 hours for *gon-14* mutants to hatch and arrest at the L1 stage. The synchronized L1 larvae were set in NGM plates. The staging was determined by examining animals under the microscope. After we synchronized the animals we started the experimental set up. One group had an initial temperature of 20°C (restrictive temperature) and the other group had an initial temperature of 25°C (permissive temperature). The control group stayed at the initial temperature throughout the course of experiment and the experimental group shifted to the opposite temperature condition after 0, 6, 12, 18, 24 hours respectively. Phenotypic data was collected two days after the shift.

Strain	**Temperature**	**% Muv**
***C.briggsae* gon-14**	25ºC	3.2%
	20ºC	96.9%
	15ºC	97.8%

Figure 4: Shows the data collected by Dr. Chamberlin of percent Muv of the *C.briggsae gon-14* worm at 25°C, 20°C and 15°C (Sharanya et al., 2015)

Results

Identification of the mutations associated with two *C.briggsae gon-14* alleles

Two candidates were selected from the genetic screen that Dr. Chamberlin did to identify Multivulva mutants. The two candidates are *Cbr-gu102* and *Cbr-gu168*. These two mutations fail to complement each other, indicating they are alleles of the same gene (Sharanya et al., 2015). *Cbr-gu102* was genetically mapped to *C.briggsae* chromosome 5 (Sharanya et al., 2015). In preliminary results, a whole genome sequencing analysis of genomic DNA from *Cbr-gu102* identified a number of mutations on chromosome 5, including one in the *C.briggsae* gon-14 gene. I sought to identify the mutation associated with the mutant allele carrying out sequencing of genomic DNA from both gu102 and gu168 strains. This sequencing demonstrated that we have two alleles for the *gon-14* gene in the lab.

Both alleles have a point mutation. The gu102 allele has a point mutation that affects the splice site at the 5' end of intron 5 (G to **A**) (Figure 5A). The gu168 allele has a point mutation that affects the splice site at the 5' end of intron 4 (C to **T**) (Figure 5B).

- The gu102 mutation is designated in red bold:

  ACAATTGAATAT**A**TACGAAAATTGTTTCAAACGAATCTGAATA

- The gu168 mutation is designated in blue bold:

  ACATGCAT**T**GACTGTTCAGTGATT

The point mutation in both alleles causes the introduction of a premature stop codon in the transcribed mRNA. The codons of the mRNA located after the stop codon are not translated because the nonsense codon in the mRNA is reached too early during the translation of the protein. This in turn causes the production of a nonfunctional protein codon.

After we identified the mutation we sought to understand how the mutation was changing the gene product (Figure 6). We predicted that the *gu102* mutation would result in a failure of intron 5 to be removed from the mRNA. Conceptual translation of the resulting sequence predicts that the mutation would cause the introduction of an intron between exon 5 and 6 and insert a premature stop codon. We confirmed the splicing defect using RT-PCR on RNA from *gu102* mutants. The results of this amplification showed that indeed the introduction of the mutation causes the inclusion of an intron between exon 5 and exon 6 in mature mRNAs.

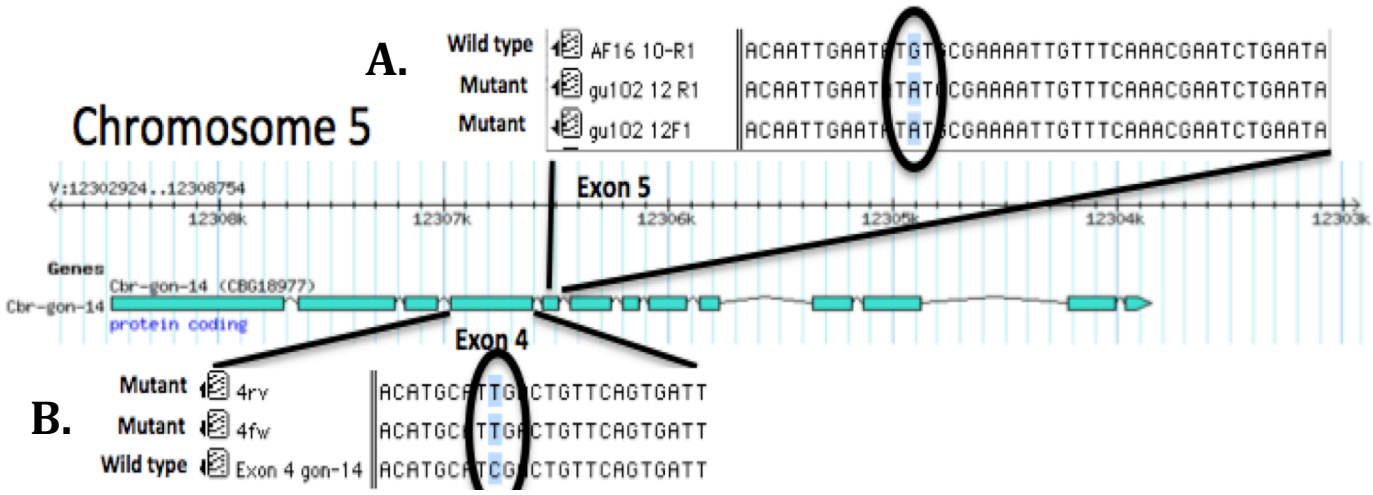

Figure 5: Shows the DNA alignment of *C.briggsae* wild type and mutant sequences. *C.briggsae* gu102 allele and its respective mutation is shown at the top-right. *C.briggsae* gu168 allele and its respective mutation is shown at the bottom-left of the figure. (Wormbase)

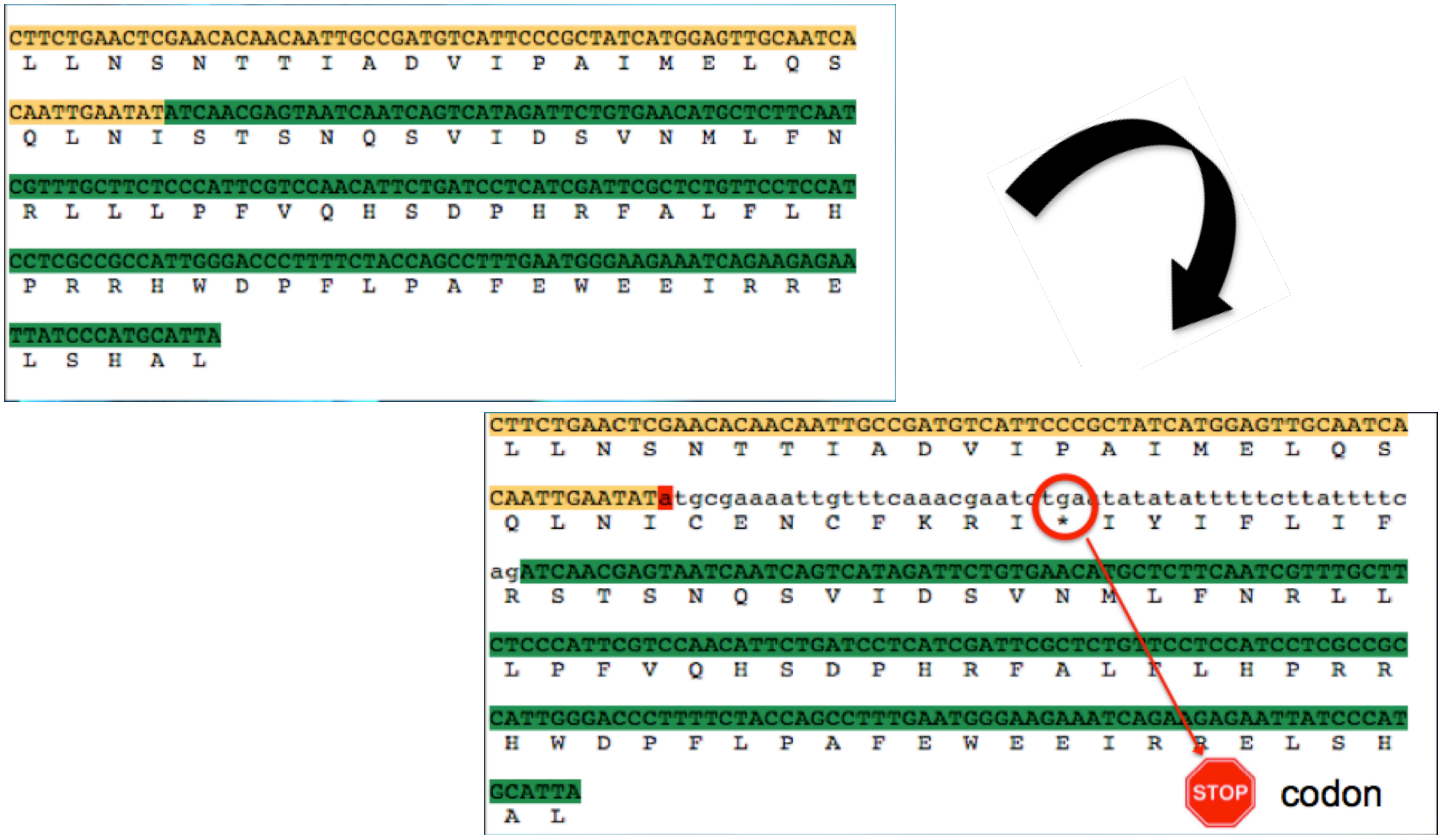

Figure 6: Shows the cDNA sequence of the C.briggsae gon-14 wild type and mutant. The picture on the left shows the normal cDNA sequencing. The picture on the right shows how the cDNA sequence changes because of the introduction of the mutation. The mutation causes the addition of an intron and a stop codon.

The temperature sensitive nature of gu102 does not result from a temperature sensitive splicing defect

RNA splicing requires complex biochemical process including the making and breaking of base pairs between RNAs (Lodish H. et al., 2000). Thus, I hypothesized that the cold sensitive nature of the *gu102* mutation might result because the intron was spliced successfully. To test this hypothesis, I purified RNA from gu102 animals raised at 20°C (restrictive) and 25°C (permissive) temperatures.

C.briggsae gon-14 animals grown at 25°C showed the splicing of intron 5 was the same for the RNA from animals grown at 20°C. These results suggest that the high temperature is not altering the expression of the mutation or the protein splicing.

- The sequence of the *C.briggsae* gon-14 animals grown at 25°C is:

  ATAT**a**tgcgaaaattgtttcaaacgaatctgaatata
  *

I also purified RNA from wild type animals raised at 20°C to confirm the *Cbr-gon-14* gene structure. cDNA amplification of the wild-type *C.briggsae gon-14* gene from animals grown at 20°C confirmed the gene structures predicted in Wormbase (www.wormbase.com) (Figure 7).

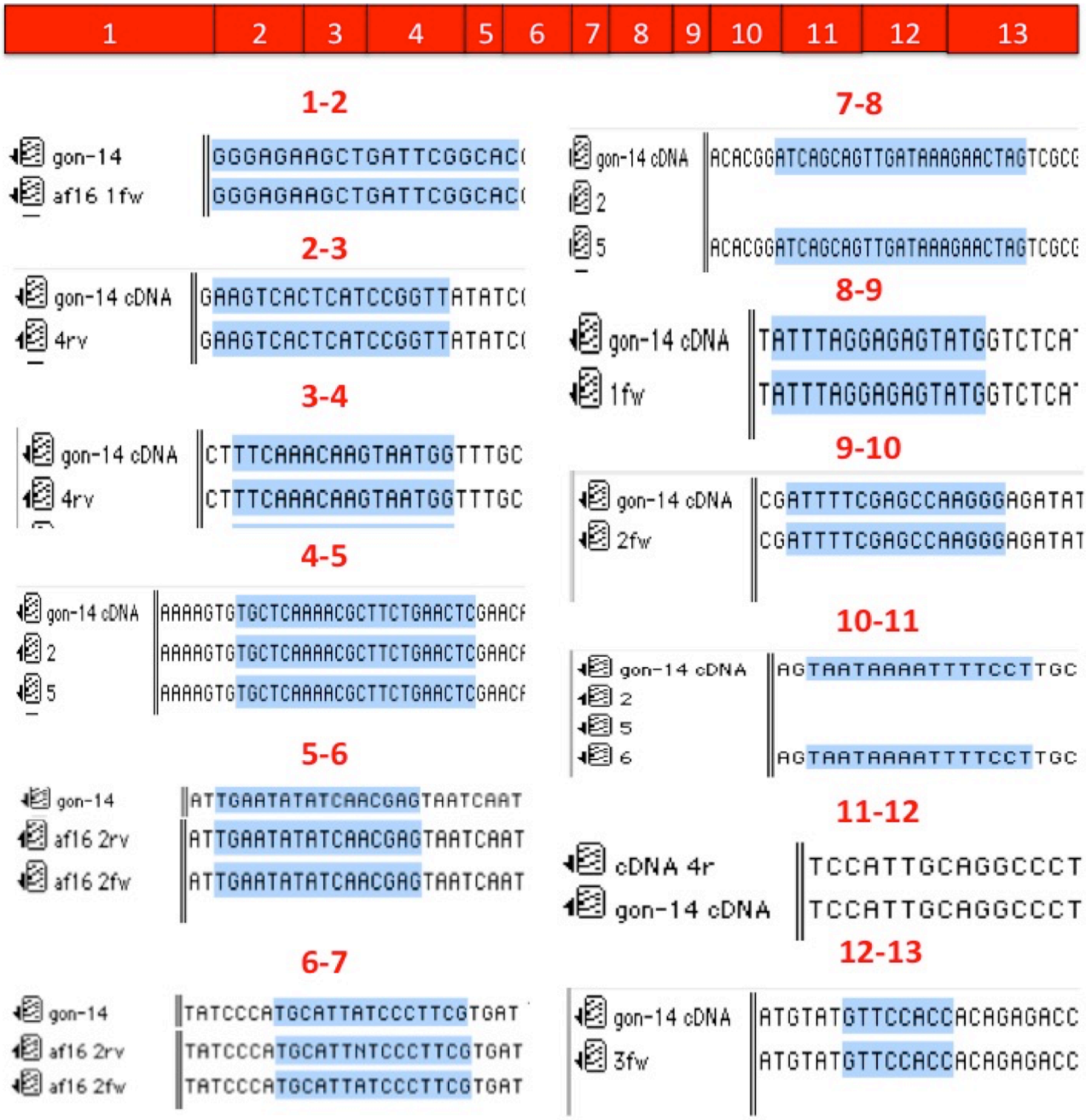

Figure 7: Shows the comparison between the wild-type and *C.briggsae gon-14* mutant sequences for all 13 exons. Red numbered row shows a model of the unspliced coding sequence of 13 exons. First column on the left shows exons 1-7 and the column on the right shows exon 7-13. Each boundary was verified with the gene structures predicted in Wormbase.

C.briggsae gon-14 mutants are temperature sensitive and the Multivulva proportion is different between worms grown at 20°C and 25°C

We hypothesized that gon-14 is temperature sensitive and it is required to prevent excessive cell division. To test this hypothesis we performed a temperature shift experiment to determine. The purpose of this experiment is to determine the temperature sensitive period of the *C.briggsae* gon-14 mutants. In this experiment we had both a control and experimental set up. In the control group, the upshift worms stayed at 25°C and the downshift worms stayed at 20°C throughout the experiment. In the experimental group, the upshift worms started at 20°C and were shifted to 25°C and the downshift worms started at 25°C and were shifted to 20°C .

The results of this experiment confirm previous studies that showed that there is a difference between the proportions Muv of the worms when grown at the two different temperatures. The results in Figure 8 show that the upshifted worms display an overall increase in the proportion Muv, whereas the downshifted worms show slight decline overtime. The results presented in Figure 9 show the same overall increasing pattern of the upshifted worms (similar to what is seen in Figure 8. However, when we look at the downshifted worms, we see a steep decrease at hour 6 but a steady increase in the proportion Muv after that.

The results of this experiment are ambiguous. Thus, to improve the results the next step would be to repeat the experiment again to corroborate and clarify the results. In addition, we should add more time points to the experiment (i.e. 28, 30, 32, 41 hours). This will give us a better perspective of the overall proportion Muv patterns overtime.

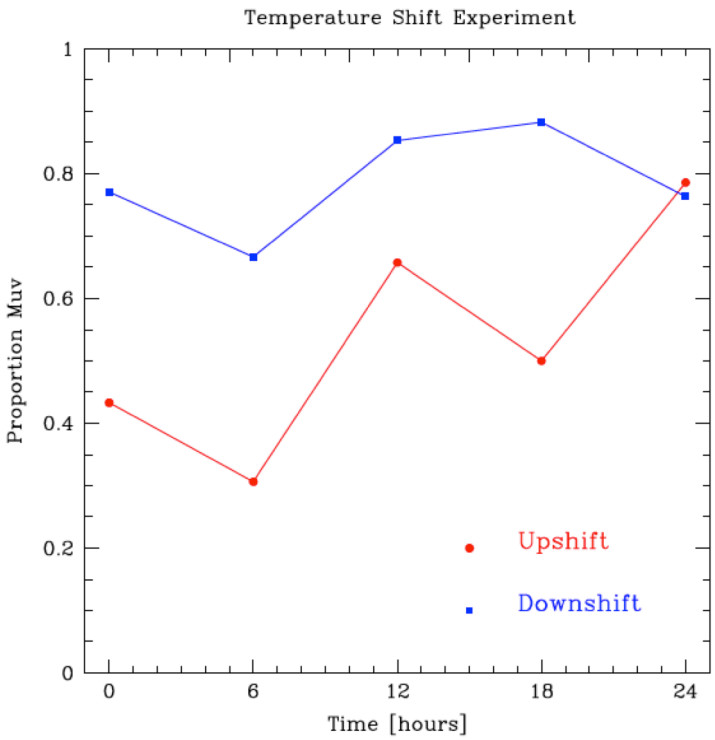

Figure 8: shows the data collected on the first trial for the temperature shift experiment.

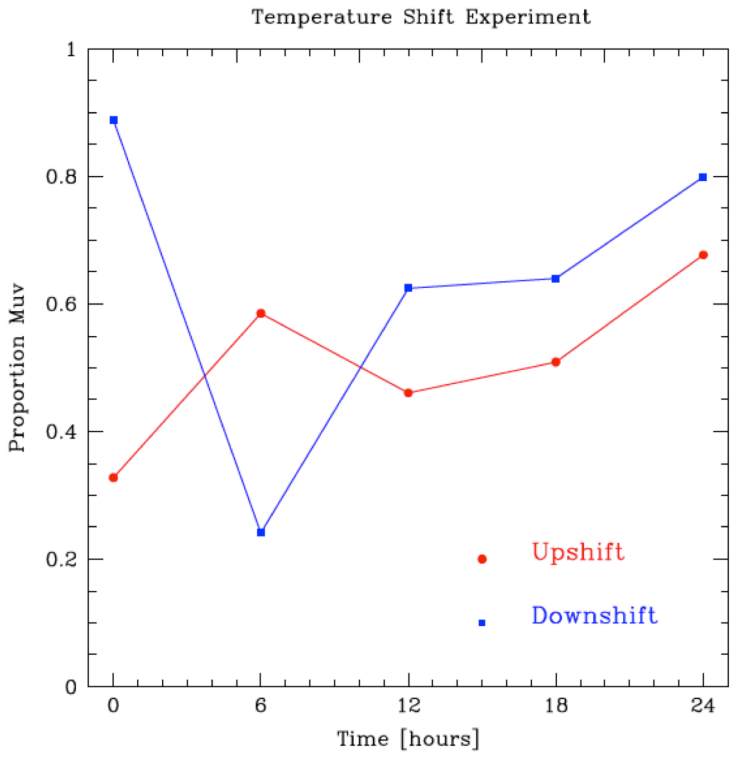

Figure 9: shows the data collected on the second trial for the temperature shift experiment.

Discussion

Tumor suppressor genes are still an enigma within the scientific community. Understanding how these genes work will help aid in the understanding of cancer development. Nematodes, such as *C.briggsae* animals are great model organisms to study tumor suppressor genes. In this work, we identified and characterized a new tumor suppressor gene called *C.briggsae gon-14* gene. There are three main points to take away from this research.

First, the mutation associated with the two *C.briggsae gon-14* alleles was identified. Sequencing results demonstrated that we have two alleles for the gon-14 gene in lab. Both alleles have a point mutation that causes the addition of an intron and the introduction of a premature stop codon. Consequently, this causes the production of a truncated protein and the protein's failure to splice.

Second, in gu102 animals, temperature is not affecting the expression of the mutation or the protein splicing. The RNA making process is very complex. Temperature could be a factor that potentially affects this process. However, when we looked at *C.briggsae gon-14* animals grown at 25°C and compared these to *C.briggsae* animals grown at 20°C we see that both showed the splicing of intron 5. These results suggest that high temperatures is not affecting the RNA making and breaking process thus, it is not altering the expression of the mutation or the protein splicing. In addition to studying the temperature sensitivity of the gu102 animals I also confirmed the C.briggsae gon-14 gene structure. We amplified wild-type *C.briggsae gon-14* cDNA from animals grown at 20°C and we confirmed the exon boundaries of all 13 exons in the gene by comparing it with the gene structures predicted in Wormbase.

Third, we verified that the *C.briggsae gon-14* mutants are temperature sensitive and that the Multivulva proportion between worms grown at two different temperatures is different. Our

results show that there is indeed a difference between the Multivulva proportion of the worms grown at two different temperatures but we could not pinpoint the specific time in development when the gon-14 gene function is required in development. However, when we compare our temperature shift experiment data to Sharanya's et, al., 2015 data we do not see quite the sharp difference in terms of proportion Muv. This could be due to many different factors including the different experimental approaches taken.

The next step to further understand this tumor suppressor gene could be a profound study of its RNA using RNA sequencing. This method will unveil the presence and quantity of RNA at a given point in development. RNA sequencing will help us understand if the gene is tissue specific and if the gene expression changes across time. Altogether, this information will help us understand how tumor suppressor genes work and their involvement in cancer development.

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Supplementary Table 1

Primers Table		
Primers	**Sequence (5' → 3')**	**Purpose**
genDNA A	AGAACCGTTGGAAGCTACGAG	DNA sequencing
genDNA B	TTGTGACTGGAAAGGCCGAG	DNA sequencing
genDNA C	GCACTCATCGAATCACGAAGG	DNA sequencing
genDNA D	GATCCGTGTTGGTAGTTCATCTTC	DNA sequencing
genDNA E	GCTCTGTTCCTCCATCCTCG	DNA sequencing
genDNA F	TTCGTCCAACATTCTGATCCTC	DNA sequencing
genDNA G	ATCCCGCGCAGGTAGTTCTC	DNA sequencing
genDNA H	AATGGGCCATGAAGTGTGACTTG	DNA sequencing
genDNA I	GTCACACTTCATGGCCCATTTC	DNA sequencing
genDNA K	GTCGTCCCGTCCAACTTCAG	DNA sequencing
genDNA L	TCTACAGTAATCTTCGCAAGCGTC	DNA sequencing
genDNA M	CTGAAGTTGGACGGGACGAC	DNA sequencing
genDNA N	CGCTTGCGAAGATTACTGTAG	DNA sequencing
genDNA O	GAATGAGAACTGGGAGAAAGTCG	DNA sequencing
genDNA P	AGGAGAAGTGATAGGGAAAGGAAG	DNA sequencing
cDNA 1	CTATTGTGACTGGAAAGGCCGAG	cDNA amplification
cDNA 2	AGAACCGTTGGAAGCTACGAG	cDNA amplification
cDNA 3	GTAGCCGTGGAAAGCGAGTC	cDNA amplification
cDNA 4	TGCTGATCCGTGTTGGTAGTTC	cDNA amplification
Exon 1-2 A	TCCATCCACATCAAAGCCGAAC	cDNA amplification
Exon 1-2 B	CCCAGAGTTGTCTGATTGTGAAG	cDNA amplification
Exon 2 A	AAATTCAACCAGATAATGGCCCAG	cDNA amplification
Exon 2 fw A	CTTTCTGGCGTTCTCTGTTC	cDNA amplification
Exon 2 fw B	TTCGACACTACATCATTCAACAATC	cDNA amplification
Exon 2 B	TCAACCAGATAATGGCCCAGTG	cDNA amplification
Exon 4 rv A	CTGGCTGACGAAATCACTGAAC	cDNA amplification
Exon 4 rv B	TGGATATTCTCCGTAGGTTCG	cDNA amplification
Exon 4 rv C	TGTTCGAGTTCAGAAGCTGG	cDNA amplification
Exon rv D	TGATAGCGGGAATGACATCG	cDNA amplification
Exon rv E	AGAAGCAAACGATTGAAGAGC	cDNA amplification
Exon rv F	GATCAGAATGTTGGACGAATGG	cDNA amplification
Exon 6 1.1	TTCCCGCTATCATGGAGTTG	cDNA amplification
Exon 6 1.2	TCAGTGATTTCGTCAGCCAG	cDNA amplification
Exon 6-7 junction	GCACATTATATCTCCCTTGGCTCG	cDNA amplification
Exon 9 fw A	TGATGTGAGCAAGGCTGTTAG	cDNA amplification
Exon 9 fw B	CCATTGACGCTTCTTTCTATTTAGG	cDNA amplification
Middle fw A	ATACTGCAATCGACCCGAAAGG	cDNA amplification
Middle fw B	TTCGAGCCAAGGGAGATATAATGTG	cDNA amplification
End rv A	ATTGGTGGTTTATGTATCTGATGTG	cDNA amplification
End rv B	CATCGTTCTGACTGCGTAATACTTC	cDNA amplification
Exon 13 rv	ATACACCTTTTTCGATGCTGCC	cDNA amplification

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